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A Mutation Causing Alport Syndrome with Tardive Hearing Loss Is Common in the Western United States

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Summary

Mutations in the COL4A5 gene, located at Xq22, cause Alport syndrome (AS), a nephritis characterized by progressive deterioration of the glomerular basement membrane and usually associated with progressive hearing loss. We have identified a novel mutation, L1649R, present in 9 of 121 independently ascertained families. Affected males shared the same haplotype of eight polymorphic markers tightly linked to COL4A5, indicating common ancestry. Genealogical studies place the birth of this ancestor >200 years ago. The L1649R mutation is a relatively common cause of Alport syndrome in the western United States, in part because of the rapid growth and migratory expansion of mid-nineteenth-century pioneer populations carrying the gene. L1649R affects a highly conserved residue in the NC1 domain, which is involved in key inter- and intramolecular interactions, but results in a relatively mild disease phenotype. Renal failure in an L1649R male typically occurs in the 4th or 5th decade and precedes the onset of significant hearing loss by ~10 years.

Introduction

Alport syndrome (AS) is an inherited disorder characterized by hematuria often leading to renal failure (MIM 301050; Alport 1927; Gregory and Atkin 1993). It may be accompanied by extrarenal manifestations including sensorineural hearing loss (Crawfurd and Toghill 1968), anterior lenticonus (Arnott et al. 1966), retinal stippling (Gelisken et al. 1988), esophageal leiomyomatosis (Cochat et al. 1988), or macrothrombocytopenia (MIM 153650; Epstein et al. 1972; Eckstein et al. 1975). After renal transplantation, some AS patients develop anti-GBM (anti–glomerular basement membrane) disease in the transplanted kidney (Milliner et al. 1982), often resulting in loss of the transplant. Defects in COL4A5, the gene encoding the α5 chain of type IV collagen, account for a substantial fraction of AS (Barker et al. 1990; Hostikka et al. 1990), with close to 100 different mutations now described (Tryggvason et al. 1993). These lesions affect basement membrane structure, causing progressive glomerular damage and, presumably, many of the extrarenal abnormalities. Clinical heterogeneity exists between different families, with respect to age at onset of renal abnormalities and of renal failure, degree of hearing loss, and other extrarenal signs. Early and severe hearing loss is usually correlated with rapid progression of renal disease. Genotype/phenotype correlations are difficult to establish, as nearly all of the known defects in COL4A5 are unique alterations found in small kindreds and the ability to assess the range of phenotypic expression of individual mutations is limited. Gene-carrier females usually exhibit hematuria, but the occurrence of end-stage renal disease (ESRD) and hearing loss is much rarer and at a later age at onset than for males (Gregory and Atkin 1993), with severity of disease expression likely related to the degree of skewing of X-chromosome inactivation in critical cell populations during development.

Subjects and Methods

Subjects

All protocols were approved by an institutional review board (IRB). The 45 families in the Iowa study either include an index case who underwent diagnostic renal biopsy and who had characteristic thickening and splitting of the lamina densa or show a history of at least one case of renal failure and hematuria with or without hearing loss in a pedigree consistent with X-linked inheritance. The Utah study has identified 76 fam-

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ilies with multiple cases of hematuria unexplained by trauma, cystic disease, IgA nephropathy, or familial thin GBM disease. Families in the Iowa study included 18 from Washington state and 27 from Iowa, while the Utah study included 39 from Utah, Idaho, Wyoming, or Montana, 17 from other states west of the Mississippi, 8 from the northeastern United States or Canada, 10 from the southeastern United States, and 2 from overseas. Five families in the Utah study (K501, K502, K1968, K2131, and K2136) were formerly described as families C, 3, H, X, and W, respectively (O'Neill et al. 1978; Hasstedt et al. 1986).

Family P (K500) is a large Utah family with adult-onset ESRD and hearing loss that has been extensively studied for nearly 50 years (Hasstedt and Atkin 1983). The mutation in K500 is C1564S (Zhou et al. 1991*a*).

Patient Data Collection

The primary sources of genealogical information were publicly available records and records kept by the families themselves. Standard questionnaires, medical records, autopsy reports, and telephone interviews were used to obtain information about hematuria, status of renal function, transplantation experience, and hearing loss. Age at onset of hearing loss was defined as the age at which the patient first perceived an impairment.

Laboratory Methods

RNA was isolated (Pruchno et al. 1991) from dermal fibroblasts maintained as described (Bonadio and Byers 1985). Primers B (5′ AAC ACA AAA GGA ATT CTT CAA AAT G 3′) and A (5′ CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CGC CCG GAT CGC AGT TCA CAG TCA GAC G 3′) were used to RT-PCR amplify a 405-bp fragment, including the cDNA encoding amino acids of COL4A5 from 1574 to the C-terminal end (Zhou et al. 1991*b*). Products were analyzed by denaturing gradient-gel electrophoresis (DGGE; Myers et al. 1985; Sheffield et al. 1989) and/or gel purified for direct DNA sequencing. Detection of mutations in COL4A5 exons 49 and 50 (Zhou et al. 1992*a*) by RNase protection was performed essentially as described elsewhere (Kaufman et al. 1990), except that target genomic DNA was first PCR amplified with primers PW2 (GGA CCT GAA TTA AAG CTA TAA GCA C) and PW3 (TGT TCC TTC TCC TTT TCC TTT ACC), designed using published intron sequences (Zhou et al. 1991*b*).

Allele-specific oligonucleotides (ASO) for the normal (5′ CTT TTG GCT GGC AAC TG 3′), and mutant (5′ CTT TTG GCG GGC AAC TG 3′) sequences, were 5′-end labeled and hybridized as described elsewhere (Wallis et al. 1990). Target segments, containing exons 49 and 50 and the intervening intron, were amplified from genomic DNA with primers A and C (5′ CTG AAT TAA AGC TAT AAG CAC 3′) or PW2 and PW3 and applied to nylon filters. After hybridization, filters were washed for 30 min in 6 × SSC, followed by 10-min washes at 48°C and then at 56°C.

Genetic markers for DXS178 (Allen and Belmont 1992); COL4A5 2B6 and 2B20 (Barker et al. 1992); DXS456 (Luty et al. 1990); and DXS1105 and DXS1059 (Gyapay et al. 1994) were assayed as described elsewhere (Barker and Fain 1993). Marker alleles for DXS17 and DXS287 were determined as described elsewhere (Kornreich et al. 1992). Detailed mapping of these markers has been reported (Barker et al. 1991; Fain et al. 1991; Willard et al. 1994).

Linkage and Statistical Methods

Pairwise analysis of linkage between AS and selected markers was performed using LINKAGE (Lathrop et al. 1984). Parameter values used were based on previous analyses (Barker et al. 1991). Age-dependent trends in the occurrence of hearing loss or ESRD were examined using a life-table method (Colton 1974, pp. 244–249), as follows. The cumulative risk for hearing loss or ESRD by age interval t is given by $Q_t = 1 - \prod(1 - q_i)$, for $i = 1$ to t, where $q_i = d_i/(O_i - w_i/2)$ is the estimated probability of hearing loss or ESRD occurring during age interval i, O_i is the number of gene-carrying males who reached the ith age interval without the symptom, d_i is the number of these who report symptom onset during the ith age interval, and w_i is the number without the symptom but not observed at ages beyond the ith age interval. An approximate value for the standard error, $SE(Q_t)$ is given by the formula $(1 - Q_t)([\Sigma\{q_i/(O_i - d_i - w_i/2)\}]^{1/2})$for $i = 1$ to t. A statistical comparison of life-table risk values Q_t and Q_t' for two different families was made by computing the statistic $z = (Q_t - Q_t')/[SE(Q_t)^2 + SE(Q_t')^2]^{1/2}$, which is distributed approximately as a unit normal. Corresponding significance levels are obtained by referring to tables of the standard normal distribution.

Results

Mutation Identification and Association with Disease

Mutations in Iowa kindreds I9016 and I9104 were detected by RT-PCR and DGGE. Determination of the cDNA sequence revealed a single T→G transversion in the second nucleotide of the codon for amino acid residue 1649 (fig. 1*A*). This change results in the substitution of leucine by arginine at this position in the translated protein. The identical mutation was independently discovered in an affected male of Utah K502 by using an RNase protection assay. A genomic PCR product was sequenced with primer PW2 to reveal the T→G change.

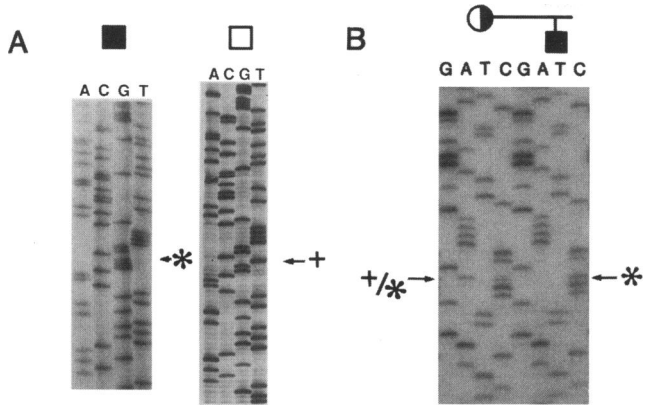

Figure 1 *A*, Nucleotide sequence of mutant (*left*) and normal (*right*) cDNA prepared from proband I9104 and control, respectively. Arrows mark the normal T (+) and mutant G (*) positions. *B*, DNA sequencing of the PCR product from a carrier female (*left*) in K2124 shows the presence of both the normal (+) and mutant (*) nucleotides, arrowed. The sequence of her affected son (*right*), contains only the mutant pattern. In panel *B*, the noncoding strand was sequenced; normal (+) is A, and mutant (*) is C.

Although kindreds K502, K501, K1968, and K2124 were ascertained through independent probands, subsequent genealogical study showed that K501, K1968, and K2124 share a common ancestor. Ancestors of K502 were geographically adjacent to these circa 1800. This history and the phenotypic similarities observed in affected males suggested the possibility of a common mutation. Sequencing of genomic PCR products from members of all three additional kindreds revealed the same T→G change (fig. 1*B*).

To test whether L1649R is a common AS mutation or an intragenic polymorphism, an ASO probe test was devised and applied to gene-carrying individuals from 121 AS kindreds, as well as 75 normal females and 10 normal males unrelated to one another. Specific hybridization to the mutant oligonucleotide was observed for nine different AS families, including all of the six known from sequence data to carry the T→G change and three others, K2120, K2131, and K2136. The 160 control chromosomes showed hybridization only to the normal oligonucleotide. Genetic linkage analysis confirmed that a COL4A5-linked mutation is responsible for the disease in kindreds K501, K502, K2120, K2131, and K2136. LOD scores for linkage to markers in the COL4A5 region were 8.45, 2.08, 1.8, 3.33, and 3.73, respectively, at zero recombination. The marker haplotype including L1649R was present in all affected males and carriers. ASO testing of a subset of members of K502 and all sampled members of K1968, K2120, K2124, K2131, and K2136 directly demonstrated the expected pattern of mutant-only hybridization in 17 affected males, normal and mutant hybridization in 30 carrier females, and normal-only hybridization in 30 unaffected males and 24 females (fig. 2). The combined results of linkage analysis, direct sequencing, and ASO testing of unrelated normal controls and members of AS kindreds confirm that L1649R is present in the disease-gene-carrying members of each of the nine families and in no other tested individuals.

Confirmation of Common Ancestry by Marker Haplotype

To test the hypothesis of a common origin of the T→G change found in these nine families, we examined the haplotype of eight genetic markers near COL4A5. Intragenic markers 2B6 and 2B20 each lie within a few kilobase pairs of COL4A5 exons (Barker et al. 1992) The five markers, DXS178, DXS456, DXS287, DXS17, and DXS1105, lie within a defined region that contains COL4A5 and has a genetic length of ~2–4 cM (Barker et al. 1991; Willard et al. 1994), while DXS1059 is ~3 cM telomeric from DXS1105 (Gyapay et al. 1994). The order of the loci is Xcen–DXS178–DXS17–(COL4A5-DXS456-DXS1105)–(DXS287-DXS1059). In eight of the nine L1649R families, an affected male carried the identical array of alleles at all tested loci (fig. 3). On the basis of estimates of allele frequencies at each marker locus, and under the assumption of no association between the alleles at different loci, the expected frequency of this haplotype is $<1.1 \times 10^{-5}$. The assumption of independence of allele types for these close markers is supported by data from normal reference families (data not shown). The chance for co-occurrence of this very rare haplotype in all of the eight kindreds is negligible ($\sim 2 \times 10^{-40}$), strongly supporting the conclusion that the variant arose on a common ancestral chromosome. In I9016, the haplotype is also identical except for DXS456 and DXS1059. Since the centromeric and intragenic markers are all identical to the ancestral type in I9016, it appears that a crossover separated the mutation site from markers telomeric to COL4A5, including DXS456, DXS287, and DXS1059. DXS287 is likely to be identical by chance, as the frequency of the 2 allele is .6.

Phenotypic Expression of L1649R: Tardive Hearing Loss

In all nine families, affected males were first noted to have microscopic hematuria in childhood and developed renal failure in adult life. In each family there is at least one affected male who has had a renal biopsy showing GBM alterations typical of AS or showing light microscopical features consistent with the diagnosis. Many of these families are notable for including one or more affected males with severe renal insufficiency but without functional hearing loss. To refine this observation,

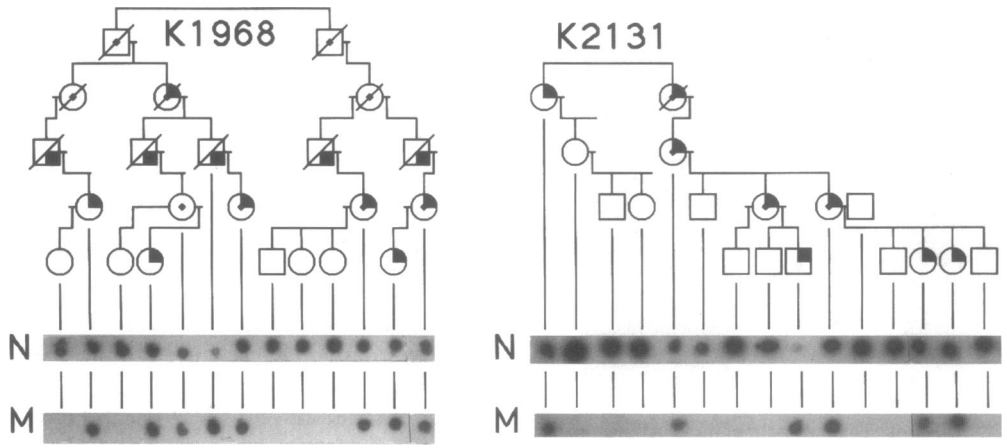

Figure 2 Allele-specific hybridization in families K1968 and K2131, with oligonucleotides corresponding to normal sequence (N) and L1649R (M). All affected males and carrier females hybridized with the M probe, while all unaffected individuals and carrier females hybridized with the N probe. Weak residual hybridization of the N probe to the mutant sequence is evident in samples of affected males; however, hybridization of the M probe is completely specific for the mutant sequence. ⊡ = Inferred gene-carrying male; ⊙ = inferred gene-carrying female; ◪ = ESRD; ◔ = hematuria; and ⊠ = deceased.

life-table analysis (Colton 1974, pp. 244–249) was applied to age-at-onset data for self-reported hearing loss and for ESRD from 87 males with the L1649R mutation. For comparison, similar data were analyzed for 54 affected males from a single large kindred, K500, previously shown to bear the C1564S mutation, which also causes adult-onset renal failure. For both kindreds, the occurrence of hearing loss (fig. 4*A*) or ESRD (fig. 4*B*) under the age of 20 years was rare. There were striking differences between the two mutations in the typical onset age for hearing loss and also in the usual sequence of onset of hearing loss with respect to ESRD. Over 70% of C1564S males reported hearing loss by age 30 years, compared with <5% of males with the L1649R mutation ($z = 7.8$; $P \ll .01$). It is most notable that for males with the L1649R mutation, 41 (77%) of 53 had *not* experienced hearing loss at the time of diagnosis of ESRD, compared with 7 (32%) of 22 for C1564S. Typically, significant hearing loss in L1649R males occurred ~10 years after the onset of renal failure (fig. 4).

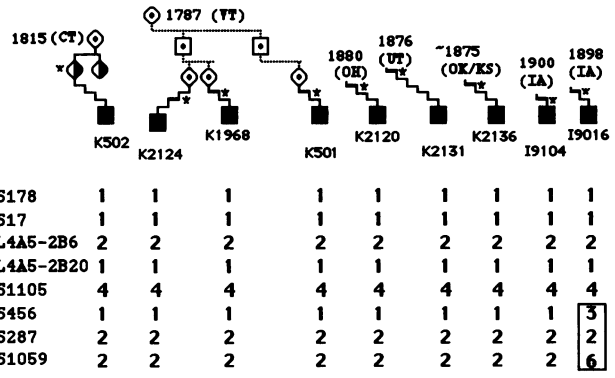

Figure 3 Common haplotype for all kindreds with the L1649R mutation. Estimates of the population frequency of each component allele, based on 45 chromosomes from unrelated, unaffected family members are ≤.02, .53, .43, .76, .73, .02, .6, and .3, for the markers in the order listed. The genealogy of each kindred is represented with symbols of individuals or by steps with each horizontal element representing one generation. ◑ = Known gene-carrying female; ■ = nephritic male; ⊙ = inferred gene-carrying female; and ⊡ = inferred gene-carrying male. A thick horizontal element and/or an asterisk (*) marks the earliest generation with a known gene-carrier female or nephritic male. The date and location of birth of the earliest known or inferred gene-carrier ancestor is also shown.

History and Geographic Distribution of L1649R Gene Carriers

Information concerning the geographic locations of living L1649R gene carriers known to our study is summarized in figure *5a*. The greatest number of cases and the greatest concentration are in Idaho and Utah. Cases occur mainly in the West, although gene carriers are resident in 14 different states. Figure *5b, c,* and *d* presents the known patterns of migration of the ancestors of each kindred. For K501, K1968, and K2124 (fig. *5b*), a common ancestor was born in Vermont in 1787 (fig. 3) and had 13 children, including two gene-carrying sons (JCT and SHT) born in New York in 1822 and 1830. Both sons migrated west, with the Mormon pioneers. SHT had four wives and 24 children, including seven obligate-carrier daughters. One of his daughters had >10 children from 1885 to 1914, including four gene carriers. She moved with her family from Utah to Idaho in 1909, and these people are the ancestors of K501. JCT was a farmer in Iowa in 1850 and in Oregon during the 1860s. One of his daughters, who

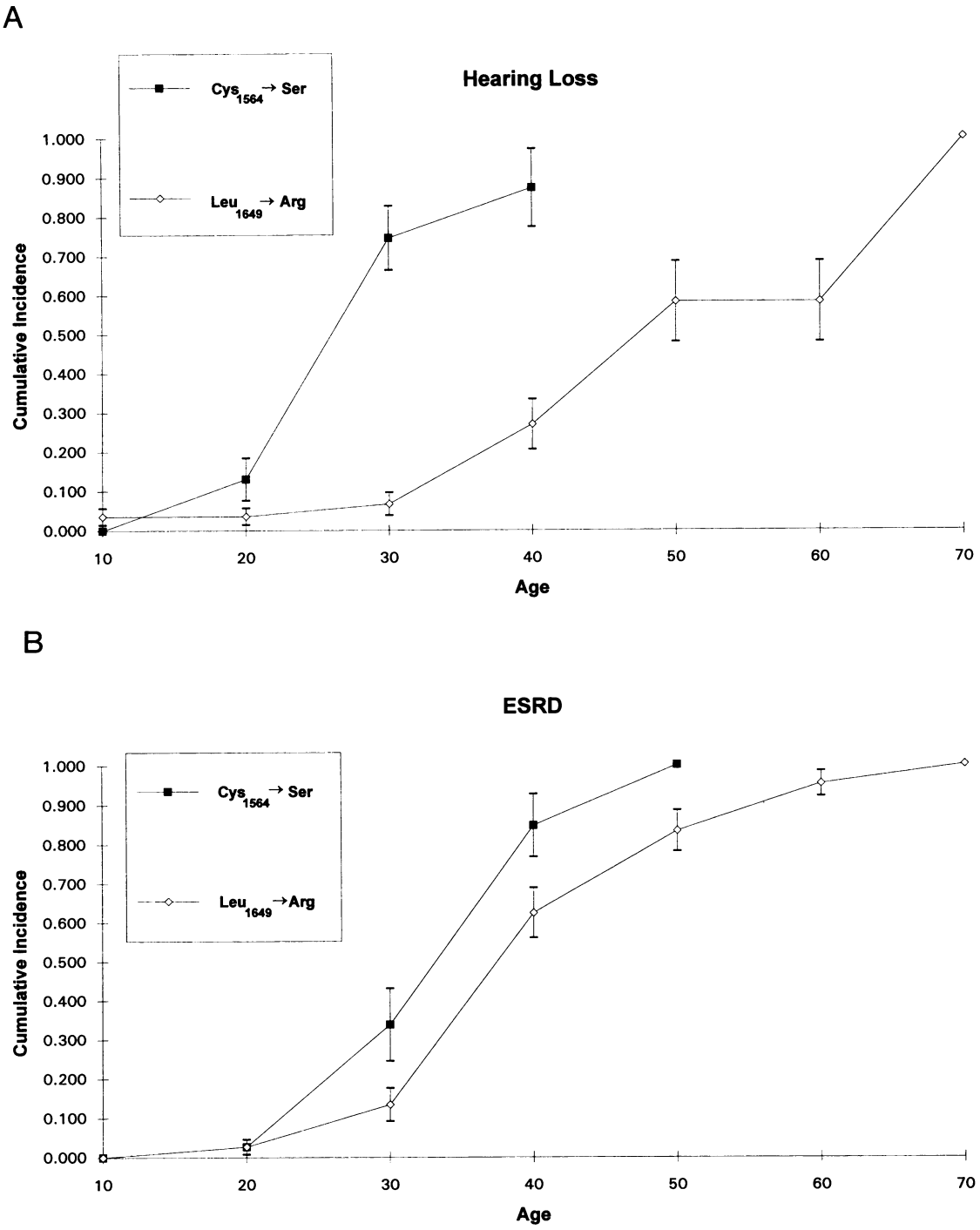

Figure 4 Age-specific cumulative incidence of hearing loss (*A*) and ESRD (*B*) for affected males with two different mutations in COL4A5, calculated as described in Subjects and Methods. Values are plotted for each age interval to which any relevant subject survived. Standard errors are shown as error bars at each point with a defined, calculable value.

was born in 1848, possibly in Illinois, is the progenitor of K2124. Her eight children were born between 1867 and 1889. In 1886, she and her family moved from Oregon to Montana, where gene-carrying descendants still reside. A second daughter of JCT, born in 1847, had 13 children between 1863 and 1883. Her family moved from Oregon to Utah in 1868. At least two of the children were gene carriers and are the ancestors of K1968. The carrier ances-

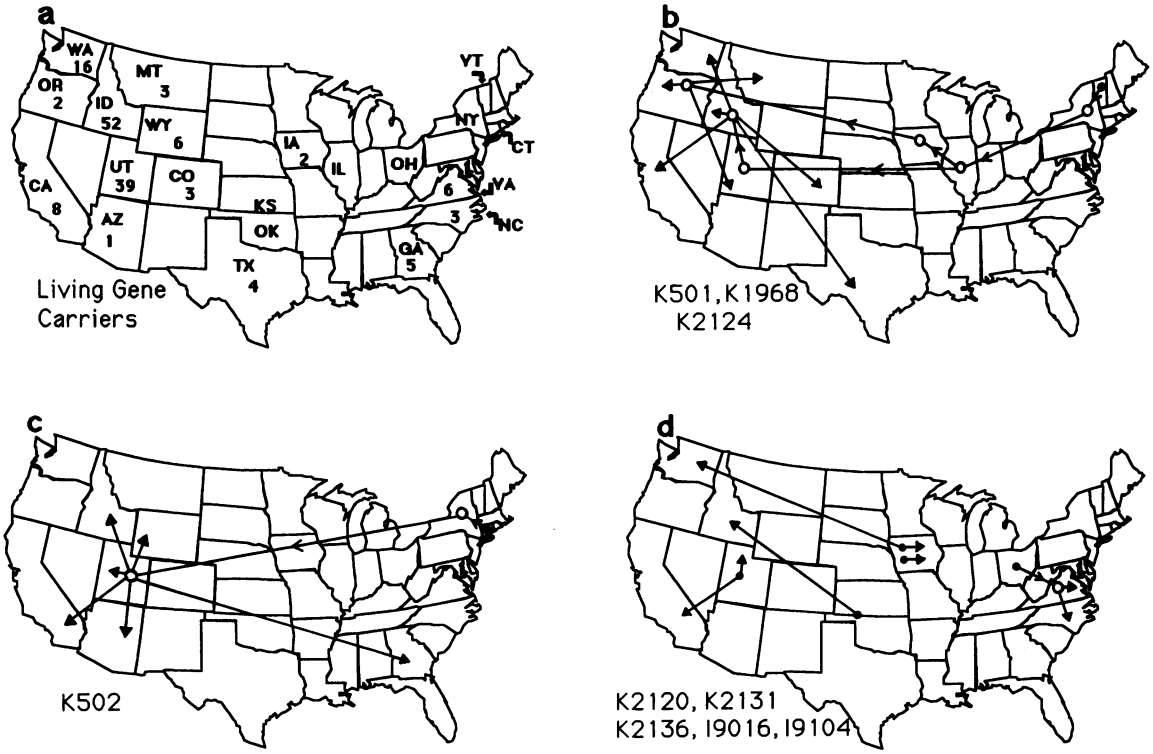

Figure 5 Current locations of known, living L1649R gene carriers (*a*) and migration patterns of known ancestors (*b*, *c*, and *d*). Panel *a* shows the current count of L1649R females and males by state. These include all persons identified by linkage or ASO testing as well as phenotypically affected persons in the direct line of descent. In panels *b*, *c*, and *d*, filled circles indicate the place of birth of the earliest gene-carrier ancestor. Open circles represent points of temporary settlement and/or sites from which further migration occurred, in the direction indicated by flow arrows (—≻—) or filled arrowheads (—▶). Filled arrowheads appear in states with currently living gene-carrying descendants.

tor of K502 (fig. 5*c*) was born in Connecticut in 1815. One of her carrier daughters (fig. 3) was born in New York and migrated to Utah, with descendants remaining in Utah and also migrating to other western states. For kindreds K2120, K2131, K2136, I9016, and I9104, less is known of migration history, and this information is summarized in figure 5*d*. The dates and locations of birth of the earliest known ancestors are as shown in figure 3. This presentation of the prevalence of L1649R is likely incomplete. Extensive genealogies have been developed for a limited number of families, and even these genealogies include many untraced branches with known or likely gene carriers. More detailed genealogical information is available from the authors, for IRB-approved studies.

Discussion

The results presented here demonstrate the occurrence of an identical alteration in the COL4A5 gene in each of nine independently ascertained AS kindreds. The L1649R change is very likely to have functional significance. It replaces a neutral amino acid with a charged basic one in a highly conserved nonpolar region of the NC1 domain (Pihlajaniemi et al. 1985; Hostikka et al. 1987; Blumberg et al. 1987; Cecchini et al. 1987; Soinen et al. 1987; Hostikka and Tryggvason 1988; Guo and Kramer 1989). The sequence change is completely associated with disease occurrence in members of nine AS families and is absent from >160 unrelated normal chromosomes. Haplotype analysis demonstrates that persons with L1649R are descendants of one ancestor with this mutation. Even if L1649R were not etiologically significant, it is still evident that the mutation must be the same in these families, because the population frequency of their shared COL4A5 region haplotype is $<7 \times 10^{-8}$, including the rare L1649R variant.

L1649R presents a distinct clinical phenotype. Renal failure typically precedes hearing loss in L1649R males by ~10 years, and the cumulative risk of developing hearing loss is just 60% by age 60. These conclusions are based on self-reported hearing loss, because this was the most reliable, uniformly available, and functionally significant measure for this large and geographically diverse population. It is unlikely that the use of this measure introduces any large consistent bias into the analysis, as shown by the contrasting results with C1564S. A

limited audiometric study (Wester et al. 1995) confirms that hearing loss is much more profound in males with C1564S than in L1649R males.

Substantial variability in the ages at appearance of ESRD and functional hearing loss exists among individuals with identical mutations (fig. 4). This variability emphasizes the fallibility of generalizations about the phenotype associated with a mutation that is observed in only a small number of AS-affected individuals (Smeets et al. 1992; Zhou et al. 1992*b*). As an example, the L1649R kindred I9104 at first seemed phenotypically distinctive because ESRD did not appear until ages 57 and 66 in the two affected males and hearing loss was noted at the ages of 51 and 58, respectively, significantly *before* ESRD. The cumulative-risk analysis indicates that the phenotype in these individuals represents one extreme of a wide range observed in all persons with L1649R. Since they both cause a late-onset form of AS, L1649R and C1564S may have a greater range of expression than is typical. However, until this point is explored further, we suggest that studies of ≥ 10 affected males are needed before the characteristic phenotype of a particular mutation and its potential variability can be ascribed with confidence. This degree of understanding and assessment of phenotypic variability is clearly of critical importance in genetic counseling and in explaining likely outcomes to patients or parents. Use of this criterion may also prevent confusion about the relative functional significance of specific molecular defects in COL4A5.

In contrast to most described COL4A5 mutations, which account for the disease in a single family, L1649R occurs in >7% of 121 studied families. On the basis of partial genealogical reconstruction for four of the families, it is possible to infer a common ancestor who lived near New England >200 years ago. The earliest known members of this genealogy in the United States became Mormons, a religion that encourages large families, apparently contributing to the rapid dispersal and growth of L1649R kindreds. This association is consistent with the observed frequent inheritance of this mutation from ancestors traced to locations near the migration route followed by the Mormon pioneers. In the mid-1800s, this group moved from New York to temporary settlements in Illinois, Missouri, and Iowa, and finally to Utah, Idaho, and other western states. It appears unlikely that the L1649R frequency we report here pertains to the entire U.S. Alport population, because the families collected by studies in Utah and Iowa (see Subjects and Methods) are probably biased toward the inclusion of the Mormon descendants. No other cases of L1649R have been published, yet it is very likely that many more than we have reported do exist, because genealogical study reveals many untraced branches that are likely to include gene-carrying individuals. Also, because of the atypical phenotype, the diagnosis of AS due to L1649R may often be missed when it occurs in one or two individuals apparently unconnected to a larger kindred. If the proportion of AS due to L1649R were as much as 1%, then the AS gene frequency of ~1/5,000 in the U.S. population (Gregory and Atkin 1993) would imply that 250 affected males and 500 carrier females with L1649R exist now. The possibility of a yet more remote ancestor with contemporary European branches of this kindred is also not ruled out. It is remarkable that this mutation appears to have had little effect on reproductive fitness, presumably because the usual age at onset is well past the typical reproductive age and similar to the life expectancy that has prevailed in much of the last 200 years.

Acknowledgments

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